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### **Laboratory Test of a Body Fluid or Tissue Sample**

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Medicine knows the term "predisposition for a disease", which means that some people have a higher probability of acquiring a specific disease than others. If the result of such a predisposition, which is often genetically induced, is a disease that does not produce clinical symptoms, then it is often the case that the corresponding disease cannot be detected at all or only with very expensive and stressful methods.

So that the execution of such expensive and stressful methods can be limited to cases exhibiting a high probability for the presence of a predisposition for a disease, it would be desirable to find an indicator for a predisposition for a disease at a low cost for the purpose of testing in the laboratory a body fluid or tissue sample.

The present invention solves this problem by detecting cis-hydroxyproline and derivatives thereof by means of quantitative analysis in a laboratory test of a body fluid or tissue sample. The present invention uses the knowledge that cis-hydroxyproline, in particular cis-4-hydroxyproline, is an endogenic substance, whose occurrence in the body in the body fluids and in the tissue depends on the predisposition for different

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diseases. This knowledge is quite surprising. Not only because to date such a dependency was not known, but also because to date it was not even known that cis-hydroxyproline is, indeed, an endogenic substance. Rather the experts assumed in the past that the body does not synthesize cis-hydroxyproline, in particular cis-4-hydroxyproline. It was only known that trans-4-hydroxyproline is formed in the human body.

The importance of the value of a laboratory test of a body fluid or tissue sample for this trans form of hydroxyproline has already been known for a long time. Hence, bone destruction, especially in the case of osteoporosis, can be confirmed and measured in detail by means of quantitative analysis.

The present invention proceeds from the recognition that the cis form of hydroxyproline, which had not been regarded to date as an endogenic substance, is a significant factor in other diseases, including in particular cancer and cardiovascular diseases.

The quantitative analysis of cis-hydroxyproline that is performed according to the present invention has proved to be not only expedient for finding an indicator for a predisposition for a disease, but also the invention can be used for a disease that already exists clinically. The quantitative analysis of cis-4-hydroxyproline in the body fluid and / or tissues can show in particular a low level of this substance, which in the prestage or early manifestation of diseases can relate to an inadequate endogenic synthesis of this substance

due to metabolic causes. Similarly the use of the present invention can detect values that are higher than the normal cis-4-hydroxyproline values and which can relate to a change in the tissue or even necrosis.

Especially important is also the determination of cis-hydroxyproline, according to the invention, for the purpose of following the course of development of the disease, a feature that is also important for the evaluation of the efficacy of the therapeutic measures and their dosing.

Therefore, the present invention comprises the detection and quantitative analysis of cis-hydroxyproline in organisms of humans and mammals, and in particular in all body fluids, like blood, urine, lymph, cerebrospinal fluid, ascites, other exudates, but also in the body tissues.

The analysis relates not only to cis-4-hydroxy-L-proline, but also to other configurations of cis-hydroxyproline, like the L and D form and a different ring positioning of the hydroxyl group. It also relates to derivatives of cis-hydroxyproline, like N-methyl-cis-hydroxyproline, and it relates to those peptide and other compounds, from which the said substances have to be eluted first for the purpose of testing.

The said compound can be determined with various known analytic methods, which include gas chromatography, column chromatography, mass spectroscopy,

HPLC [= high performance liquid chromatography] method, ion exchange chromatography, immunoassay, radio immunoassay, enzyme immunoassay, fluorescence immunoassay and others. In the case of cleavage of peptide and similar compounds, the use of conventional conservative methods must be considered.

A preferred method, which can be used within the scope of the present invention to determine cis-hydroxyproline and its derivatives in a body fluid or tissue sample, is disclosed in claim 4. Preferred further developments can be inferred from the dependent claims referring to claim 4.

According to the method, disclosed in claim 4, to determine cis-hydroxyproline and its derivatives in the body fluids and / or tissues, the sample to be analyzed is prepared to eliminate disturbing substances; and the cis-hydroxyproline and its derivative content is determined quantitatively in this sample. The definition of the term "disturbing substance" is based on the analysis method that is used. Should the HPLC method be used, the disturbing substances are those that can falsify the measurement of cis-hydroxyproline and its derivatives based on a corresponding absorptivity at essentially the same retention time. Such substances must be eliminated. Hence, it is possible to determine cis-4-hydroxyproline, for example, in a concentration of < 1 µg / ml that is present in the human body.

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Especially preferred is the determination of the cis-hydroxyproline content in the body fluids and / or tissues by means of comparison with an external and / or internal standard. Thus, it is possible to quantify reliably the concentration of cis-hydroxyproline, even in the aforementioned small concentrations.

To determine the cis-4-hydroxyproline content using the HPLC method at an excitation wavelength of 471 nm, cis-3-hydroxyproline (cis-3-HYP) has proved to be a suitable internal standard. Using a suitable separating column (for example, RP 8) and an optimal measuring temperature (for example, 60 degrees C), its retention time (6.00 min.) is longer than that of cis-4-hydroxyproline (4.60 min.) and trans-4-hydroxyproline (3.30 min.).

~~Preferably the sample to be analyzed is prepared in several steps. An internal standard (s.o.) is added to the body fluid and / or the tissue; the mixture, obtained according to this step, is hydrolyzed. Then at least one alkali hydroxide and at least one alkali carbonate are added to the hydrolyzed mixture. The product from this step is treated with a reagent eliminating the disturbing substance; and finally the cis-4-hydroxyproline and its derivative content is analyzed quantitatively. In this respect, it has proved to be advantageous that the hydrolysis in the presence of a strong mineral acid, in particular hydrochloric acid, occurs at a raised temperature, in particular preferably at a temperature of approximately 100 degrees C. Similarly it is especially~~

advantageous, if the pH of the hydrolyzed mixture is adjusted to a value ranging from 8.5 to 9.0. Hydroxides, carbonates and / or bicarbonates of sodium or potassium can be used preferably as the basic alkali metal compounds that are appropriate for the invention.

In particular, compounds, containing keto groups, are suitable as the reagent, with which the disturbing substances are removed. In this respect, aldehydes, in particular ortho phthaldialdehyde at a concentration ranging from 45 to 55 g/l, in particular at a concentration of 50 g/l, have proved to be suitable as the keto compounds. After the solution has been allowed to stand at room temperature, any precipitate that might have formed is removed by separation by means of sedimentation, centrifugation, filtration or another suitable method.

Then an aqueous phosphate buffer and a derivatization reagent, with which the cis-4-hydroxyproline to be determined is chemically modified, are added as the mobile solvent to the solution, which is obtained above and is freed of any precipitate. Typical derivatization reagents, which are also known in the state of the art, are azo dyes, whereby dabsyl chloride at a concentration ranging from 220 to 270 mg/l, in particular at a concentration of 242.8 mg/l, is especially interesting. To accelerate the reaction, the mixture comprising the sample to be analyzed, the buffer and the derivatization reagent is heated in an advantageous manner for a short period (for example, 15 minutes) to temperatures of approximately 70 degrees C.

If necessary, the reaction solution, obtained in the preceding step, is cooled down to room temperature and optionally a small quantity of the same aqueous phosphate buffer that was added in the preceding step is added as the mobile

solvent. Then the quantitative analysis of cis-4-hydroxyproline and its derivatives is carried out with a sample, which is prepared in such a manner, by means of HPLC.

The invention is explained in detail below with the aid of the examples.

Example 1:

Preparation of external / internal standard solutions to quantify cis-4-hydroxyproline in body fluids

a) Preparation of an external standard solution: cis-4-(L)-HYP, trans-4- (L) -HYP, cis-3- (DL) - HYP

- 1) Stock solution:  $c = 0.1 \text{ mg/ml}$ ; (identical volumes of a solution of 5 mg cis-4-HYP and 5 mg trans-4-HYP in 25 ml superpure water (bidistilled water) and a solution of 5 mg cis-3-HYP in 25 ml superpure water are mixed)
- 2) mix 1 ml HCl (6 M) and 1 ml of 1)
- 3) mix 1 ml of 2) with 5 ml NaOH (0.5 M) and 5 ml  $\text{Na}_2\text{CO}_3$  (0.25 M)
- 4) adjust pH value to 8.5 by adding HCl (32%, 10 M)
- 5) mix 10 ml of 4) with 15 ml dabsyl chloride ( $c = 242.8 \text{ mg/l}$ ), heat to 70 degrees C for 10 minutes

- 6) allow to cool to room temperature, then fill up to 25 ml with acetone (should turbidity develop, a couple of drops of superpure water are added until dissolution)
  - 7) 25 ml of 6) are filled up to 146.156 ml with superpure water.
- b) Preparation of an internal standard solution (IS): cis-3-(DL)-HYP  
Identical parts by volume of the solution of cis-3-HYP (5 mg in 25 ml superpure water), used above, and superpure water are mixed.

Example 2:

Quantitative analysis of cis-4-HYP in urine:

- 1) 1 ml HCl (10 M) and 0.1 ml IS, according to example 1, are added to 1 ml urine to be analyzed and hydrolyzed at 100 degrees C for 16 hours.
- 2) 0.25 ml of an aqueous solution of NaOH (16 M) and Na<sub>2</sub>CO<sub>3</sub> (4 M) respectively are added to 1 ml of 1). Optionally the pH value is adjusted with 10 M HCl to a pH ranging from 8.5 to 9.0.
- 3) 0.5 ml of 2) is mixed intimately with 0.2 ml ortho-phthaldialdehyde (c = 50 g/l) and with 0.2 ml superpure water and allowed to stand for 60 minutes. Then any precipitate that may have accumulated is centrifuged off.
- 4) 0.2 ml of an aqueous phosphate buffer (22 % by weight acetone nitrile, 78 % by weight superpure water, di-sodium

hydrogen phosphate dodecahydrate, citric acid, pH = 4.7) and 0.2 ml dabsyl chloride ( $c = 242.8$  mg/l) are added to 0.15 ml of the supernatent solution of 3); the reaction vessel is tightly closed and heated to 70 degrees C for 15 minutes in the oven.

- 5) The reaction solution of 4) is cooled to room temperature, and then another 0.3 ml aqueous phosphate buffer is added as the mobile solvent.
- 6) HPLC measurement using an RP 8 separating column at a measurement temperature of 60 degrees C in the column oven and an excitation wavelength of 471 nm: injection volume: 20  $\mu$ l

Urine samples of 10 male and 10 female subjects of different ages were tested.

The following table lists the measurement results and the urea, creatinine and total protein data of the respective sample.

Table 1: Chromatographic Quantification of cis-4-HYP of Native Urines.

X <sup>a</sup>	Age (Y), Sex	Urea (mg/dl)	Creatinine (mg/dl)	U/CSF (mg/dl)	c(trans-4-HYP) ( $\mu$ g/ml)	c(cis-4-HYP) ( $\mu$ g/ml)
51	62, f	675 T	34.41	< 2	6.7	0.4
52	56, f	1628	71.42	3.1	8.6	0.4
53	19, f	1360	141.81	15.1	38.0	1.3
54	6 days, f	130 T	12.90	7.6	110	1.8
55	49, f	410 T	13.13	10.4	6.8	0.2
56	86, f	474 T	23.40	< 2	4.9	0.3
57	56, f	1726	105.35	9.4	24	1.2
58	83, f	763 T	48.03	5.5	5.8	0.5
59	33, f	802 T	80.23	2.6	22.8	0.3
60	65, f	957	16.34	< 2	12.6	0.2
I <sup>b</sup>					8.4	0.6
II <sup>b</sup>					9.4	1.4
III <sup>b</sup>					8.9	11.6
61	35, m	1866	151.27	4.6	26.9	0.5
62	69, m	2480	51.57	5.3	22.6	0.9
63	81, m	2197	91.77	4.8	45.0	1.5
64	86, m	2068	130.50	7.8	24.5	1.7
65	50, m	2221	194.16	9.1	31.2	0.9
66	60, m	772 T	120.69	5.4	20.3	0.8
67	57, m	2597	171.67	13.4 H	25	0.7
68	45, m	1375	70.94	< 2	7.6	0.5
69	35, m	351 T	74.06	12.5 H	17	0.2
70	69, m	337 T	75.23	6.8	5.9	0.2
I <sup>b</sup>					8.6	0.3
II <sup>b</sup>					7.9	4.5
III <sup>b</sup>					8.6	11.1

a) sample - number - EOZO - X

b) control urine I - III

I:  $c(\text{cis-4-HYP}) = 0.3 \mu\text{g/ml}$ II:  $c(\text{cis-4-HYP}) = 1.4 \mu\text{g/ml}$ III:  $c(\text{cis-4-HYP}) = 11.2 \mu\text{g/ml}$ I - III:  $c(\text{trans-4-HYP}) = 8.7 \mu\text{g/ml}$